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Evaluation of Forty New Phenothiazine Derivatives for Activity Against Intrinsic Efflux Pump Systems of Reference *Escherichia coli*, *Salmonella* Enteritidis, *Enterococcus faecalis* and *Staphylococcus aureus* Strains

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Abstract. *Background: Because phenothiazines inhibit efflux pumps of bacteria, forty new phenothiazine derivatives were tested for their inhibition of the efflux pump systems of Gram-positive and Gram-negative pathogenic bacteria. Materials and Methods: Detection of efflux pump activity was conducted by a previously described automated fluorimetric method. Results: Although many of the compounds significantly inhibited efflux by distinct bacteria, four compounds had exceptional activity against the efflux pump systems of the pathogenic wild type bacteria Escherichia coli, Salmonella Enteritidis, Enterococcus faecalis and Staphylococcus aureus. These four compounds were then evaluated for ability to reduce or reverse resistance of multi-drug resistant members of Escherichia coli, Salmonella and Staphylococcus aureus whose MDR phenotypes are mediated by specific over-expressed efflux pumps. One of the compounds, 2173, significantly reduced resistance of MDR Staphylococcus aureus. Conclusion: These results suggest possible use of compound 2173 for therapy of infections caused by this organism.*

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Multi-drug resistant (MDR) bacterial clinical isolates owe their MDR phenotypes to the over-expression of efflux pumps (1). The intrinsic efflux pump system of Gram-negative (2-5) and Gram-positive (6, 7) bacteria, which contribute to the intrinsic resistance of these organisms, when over-expressed renders the organism even more resistant to representatives of two or more distinct antibiotic classes. How resistance develops in a patient has been suggested to result from prolonged exposure to increasing concentrations of a single antibiotic, which at first promotes increases in the activity of genes that code for regulators and transporters of a given bacterium (2, 3, 8). When this is followed by exposure to a constant concentration of the same antibiotic, this results in the gradual reduction of the activity of the gene that codes for the transporter to levels expressed by the non-antibiotic exposed control (wild-type), with concomitant development of resistance to many antibiotics (3). Whereas the increased resistance to the antibiotic following serial exposure to increasing concentrations of a given antibiotic is reversible, that which results from serial exposure to a single concentration is not, suggesting the presence of mutated targets (3). These results suggest that while a patient is treated for prolonged periods with a single antibiotic, the response of the bacterium evolves initially from protection from the antibiotic by substantially increasing its capacity to extrude the antibiotic, and, when the dose of the antibiotic is maintained for long periods of time, the bacterium makes a "decision" to mutate many antibiotic targets, rendering the bacterium a truly MDR pathogen. This MDR pathogen pays a survival cost since it cannot compete successfully with its wild-type counterpart when co-cultured (3).

The aforementioned results also suggest that evaluation of a clinical isolate for its antibiotic susceptibility profile will render results that reflect the point of evolution of resistance; therefore, initially, over-expression of efflux pumps is readily demonstrable, and with time, their activity is reduced when mutations begin to appear and accumulate. Evaluation of efflux pumps of MDR clinical isolates and their response to agents that are being studied for potential efflux pump inhibitory (EPI) capacities may not yield information that is completely related to the efflux pump system. To this extent, MDR clinical isolates that are shown to over-express an efflux pump rarely assume an antibiotic response characteristic of its wild-type counterpart when cultured in the presence of an EPI.

Given these apparent facts, we have chosen to use reference strains representative of the four most frequent bacterial pathogens for the evaluation of a series of phenothiazine derivatives for inhibitory activity against their efflux pump systems. Because the members of this series of phenothiazines differ from each other by slight modification of structure, it would be possible to perform quantitative structure-activity relationships (QSARs) that would make it possible to identify the component(s) of the phenothiazine structure responsible for its EPI activity. Because the method and system used for the evaluation of EPI activity is one that provides a real-time assessment of EPI activity under physiological conditions that lend themselves to manipulation (9-14), the *in vitro* activity of the EPI may provide further insight into the interaction between the EPI and the intrinsic efflux pump of the organism.

Materials and Methods

Materials. Mueller-Hinton (MH) powder was purchased from Sigma (Madrid, Spain) and used for the preparation of agar and broth. Ethidium bromide (EB) and the EPI thioridazine (TZ) were purchased from Sigma. Forty compounds evaluated for activity against efflux pump systems of bacteria were derived from a parental phenothiazine and the derivatives were made by chemical manipulation. The phenothiazine derivatives were dissolved in dimethyl sulfoxide (DMSO). The preparation of the particular phenothiazine derivatives identified by code in this study will be reported elsewhere pending protection of intellectual property rights.

Bacteria employed. Wild-type *Escherichia coli* K-12 AG100 strain (argE3 thi-1 rpsL xyl mtl delta (gal-uvrB) supE44) (14) was kindly provided by Hiroshi Nikaido, Department of Molecular and Cell Biology and Chemistry, University of California, Berkely, CA, USA; K-12 $AG100_{TET}$ and K-12 $AG100A_{TET}$ strains were derived from their parental strains K-12 AG100 and K-12 AG100A (resistance insertion marker, *Δ*acrAB::Tn*903* Kan^r) (15, 16) and induced to high level resistance to tetracycline (Minimum inhibitory concentration (MIC) 12 mg/L) by gradual exposure to the antibiotic (2). K-12 $AG100_{TET}$ and K-12 $AG100A_{TET}$ over-expressed AcrAB and AcrEF efflux pumps, respectively (8). Wild-type ATCC 29212 *Enterococcus faecalis* (7); wild-type ATCC 25923 *Staphylococcus aureus* (6) and the progeny of methicillin-resistant *Staphylococcus aureus* (MRSA) COL_{OXA} strain induced to extremely high level resistance to oxacillin by gradual exposure to the antibiotic (6) and over expressed the NorA efflux pump (6, 17); *Staphylococcus aureus* HPV-107 strain has the QAC efflux pump (18); NCTC *Salmonella* Enteritidis (19); *Salmonella* Enteritidis 104_{CIP} (20) and *Salmonella* Enteritidis 5408_{CIP} that were derived from parents gradually exposed to ciprofloxacin achieving a high level of resistance to the antibiotic, part of which was due to the over-expression of the AcrAB efflux pump (19).

Methods. Bacteria were cultured on MH agar and incubated overnight; a single colony was transferred to MH broth incubated at 37˚C for 16 h and the MIC of all compounds against the bacteria employed in this study were determined by micro-broth dilution as per CLSI guidelines (21). The MIC of compounds to be evaluated for effects on the efflux pump system is critical for it provides the amount of compound which yields 50% the MIC, a relative concentration which is known to have little or no effect on the viability and replication of the bacterium (8).

Detection of efflux pump activity in Escherichia coli AG100. This was conducted by a semi-automated fluorimetric method previously described (9, 10). Briefly, the method follows the real-time accumulation of EB by the bacterial population with the aid of the Rotor-Gene 3000™ thermocycler (Corbett Research, Sydney, Australia) programmed for 30-40 cycles of 1 min each for a duration of 25 min at a constant temperature of 37˚C. Bacteria are first grown in MH broth until they reach an optical density (OD) of 0.6 at 600 nm. The cells are then centrifuged, washed twice with phosphate-buffered saline (PBS) at pH 7.4, and the OD adjusted to 0.6 with PBS (pH 7.4) containing 0.4% glucose, and aliquots of 0.045 mL transferred to microtubes of 0.2 mL volume. Immediately, aliquots of 0.045 mL of saline pH 7.4 containing EB and glucose to yield final concentrations of 1 mg/L and 0.4%, respectively, with and without varying concentrations of each compound to yield ½ of their MIC value, were added. The concentration of DMSO in the assay was not higher than 10%. The instrument was started and real-time accumulation of EB (amount of relative fluorescence emitted) followed up to 25 minutes, using excitation and emission wavelengths of 535 nm and 586 nm, respectively. The difference between the amount of fluorescence of compounds containing tubes and control (no compound) at the end of 25 min provided an estimation of the effect of the compound on accumulation of EB. Previous studies amply demonstrated that the increased accumulation of EB promoted by a compound over that of the control is a measure of the effect of the compound on the efflux pump system of the bacterium (9-12). Arbitrarily, agents that promote accumulation of EB by at least two-fold over that taking place in the control at the end of 25 min of assay are considered to be significantly active and hence and used for further investigations.

Whether the degree of accumulation of EB noted is due to a direct inhibition of the efflux or due indirectly to the effects on energy sources, *etc*., cannot be determined from this method alone. However, if the agent is to inhibit efflux (*i.e*. promote accumulation) then it should be able to render the bacterium more susceptible to antibiotics to which it was initially resistant due to its over-expressed efflux pump system. In order to determine whether an agent that significantly promotes accumulation renders the bacterium more

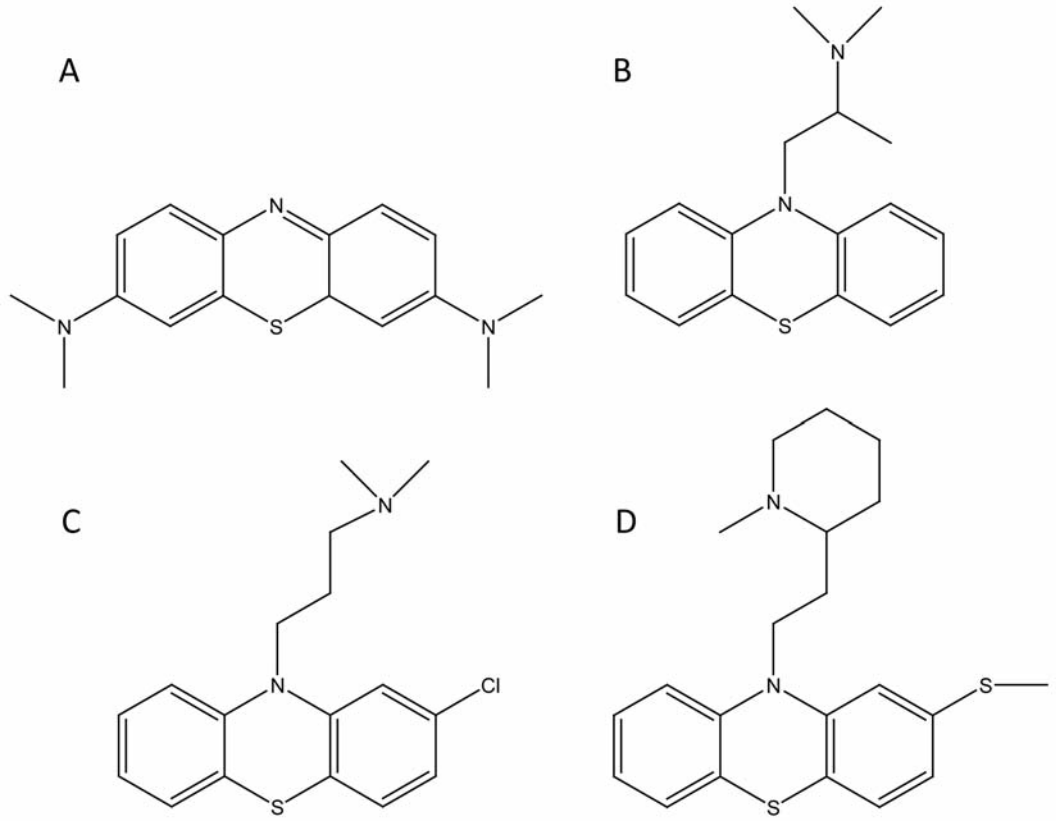

Figure 1. *Structures of Pheothiazines: A. Methylene Blue; B. promethazine; C. chlorpromazine and D. thioridazine.*

susceptible to given antibiotics, the effect of the agent on the MIC of a given antibiotic was determined as follows: firstly, an MIC of a given antibiotic is determined and then the selected agent at a concentration of equal to or less than ½ its MIC, is added in combination with decreasing concentrations of the antibiotic that range from its MIC to zero. For an agent to be considered an inhibitor of efflux, it must reduce the MIC of the antibiotic by at least two-fold (22). The use of an agent that is known to reduce the MIC of a bacterial species that over-expresses its efflux pumps served as a positive control. To this end, the phenothiazine TZ, a proven inhibitor of efflux pumps of *Escherichia coli* (9-11, 14), *Enterobacter aerogenes* (13), *Salmonella* Enteritidis (14), *Staphylococcus aureus* (6, 18) and *Enterococcus faecalis* (7, 23, 24), served as the positive control. The structures of thioridazine and other phenothiazines from which it was derived, and which also have EPI activity, are presented in Figure 1.

Determination of the ability of compounds that presented with EPI-like activity to reduce the MIC of antibiotics whose resistance was mediated by over-expression of efflux pumps was conducted as follows (2, 4, 18): The MIC for the selected compounds was first established for each of the pathogenic bacteria. Concentrations of each compound at ¼ and ½ its MIC were then added to cultures of the bacteria that contained increasing amounts of the antibiotic to which they were initially resistant. At the end of 18 h, the cultures were visually examined and cultures which had no visible turbidity indicated the MIC of the antibiotic.

Results

Graphical presentation of the effects of forty compounds derived from a parental phenothiazine at a concentration equivalent to ½ their MIC against four pathogenic bacteria on the accumulation of EB by these bacteria is beyond the limits of any journal. Consequently, Figure 2 serves to illustrate the type of data generated by the method employed in this study. The example is given for the *Staphylococcus aureus* ATCC strain. From this data, three conclusions may be made: i. The control bacterium does not appreciably accumulate EB during the period of 25 min of the assay in PBS (pH 7.4) containing 0.4% glucose and 1 mg/L of EB as previously demonstrated (4, 9-11); ii. The final concentration of DMSO of 10% does not cause a change in the rate of accumulation of EB; iii. Whereas compounds 1930 and 2115 marginally increased accumulation of EB, compound 2173 promoted a five-fold increase of accumulation. The data presented is for one of each set of duplicates of the assay and the variation between duplicates was not more than 5%.

The effect of each compound on the accumulation of EB by the pathogenic bacteria employed in this study may be calculated by obtaining the difference between the average

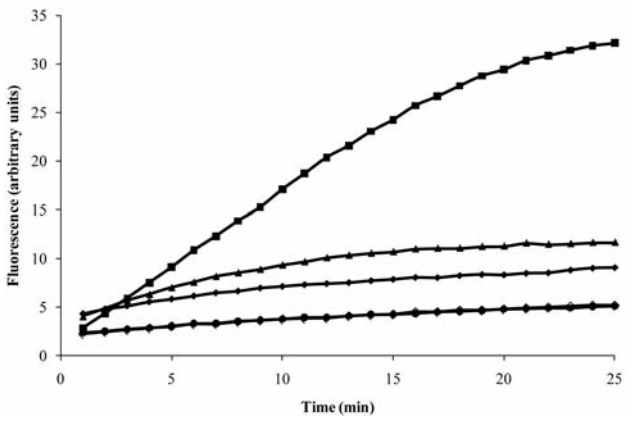

Figure 2. *The effects of phenothiazine derivatives on the accumulation of ethidium bromide by Staphylococcus aureus ATCC 25923. Accumulation of EB by Staphylococcus aureus ATCC 25923 strain was incubated at 37˚C for 25 minutes in PBS (pH 5) containing 1 mg/L of EB in the following conditions: control (◆), 10% DMSO (●), and phenothiazine derivatives 1930 (◇), 2115 (▲) and 2173 (■) at ½ of their minimum inhibitory concentration. Accumulation of EB was automatically monitored on a real-time basis.*

accumulation in the presence of the compound at ½ its MIC and that of the average control. These differences are summarized by Table I; compounds that inhibit accumulation of EB are highlighted in the Table I. Briefly, it may be noted that although many compounds inhibited accumulation of EB, with the exception of 2158, no other compound had significant activity on the accumulation of EB by each of the bacterium employed in this assay. With respect to *Escherichia coli*, compounds 2158 and 2173 significantly increased accumulation of EB. With respect to *Salmonella* Enteritidis, only compound 2158 had activity. With respect to *Enterococcus faecalis* compounds 2158, 2167 and 2166 promoted significant accumulation; with respect to *Staphylococcus aureus*, compounds 2173, 2166, 2158, 2167, and 2164 had major effects on accumulation, and a number of compounds had moderate effects considered to be significant (example-2178, 2174, 2115, 1821, *etc*.). The data of Table I suggest that *Staphylococcus aureus*, a representative of the Gram-positive group, is very sensitive to many of the phenothiazine derivatives, whereas *Enterococcus faecalis*, another representative of the Gram-positive group, is far less sensitive.

In order that an agent is considered to be a true inhibitor of an efflux pump it must be able to reduce the resistance of the bacterium to a given antibiotic to which it was initially resistant due to its over-expressed efflux pump system. The most active agents of Table I against the efflux pump system of wild-type strains of *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* Enteritidis were selected and evaluated for their ability to reduce resistance of bacteria that over-express a specific efflux pump (2, 5, 25). For this purpose we selected *Escherichia coli* $AG100_{TET}$ that over-expresses the AcrAB efflux pump by 16 times that of its tetracycline sensitive parent and thus has high level resistance to

Table I. *The effect of phenothiazine derivatives on the accumulation of EB by bacteria.*

Compound ID	*Enterococcus faecalis*	*Salmoella* Enteritidis	*Escherichia coli*	*Staphylococcus aureus*
819	4.02/50	–1.06/50	**6.00/50**	3.91/25
1821	**7.18/50**	0.13/50	**5.06/50**	**9.67/50**
1930	4.63/50	1.57/12.5	2.62/50	**22.83/50**
1931	2.64/50	0.05/50	–0.73/50	**5.21/50**
1932	3.54/50	0.00/50	–1.53/50	–2.89/50
1936	1.25/50	0.22/12.5	3.45/50	**15.01/50**
1960	0.77/50	0.24/50	–1.57/50	4.59/50
1993	8.81/50	–1.40/50	0.41/50	6.60/50
1994	4.22/50	0.01/50	**7.87/50**	**5.27/50**
1995	–0.26/ 5	–0.25/5	–0.30/5	2.95/5
1997	1.73/5	–0.13/5	–0.22/5	2.63/5
1998	0.57/5	–0.05/5	1.08/5	–1.75/5
1999	–2.22/50	–0.58/50	0.77/50	**15.13/12.5**
2000	–0.15/5	0.23/5	–0.56/5	–3.32/5
2001	3.99/50	–1.14/50	1.45/50	1.60/25
2024	–0.74/50	–1.17/25	0.19/50	**6.45/50**
2115	4.92/50	–0.41/50	**8.13/50**	**10.70/25**
2155	–0.88/50	–0.21/50	–0.03/50	**7.37/50**
2156	–0.74/50	–0.90/50	1.07/50	1.92/25
2158	**51.12/50**	**8.39/50**	**42.36/50**	**28.05/50**
2159	–0.59/6.25	–0.31/25	–0.12/50	–1.16/3.2
2160	–0.14/50	–0.15/50	0.19/50	**5.51/50**
2161	4.75/50	–1.35/12.5	**5.35/50**	1.41/50
2162	0.89/12.5	0.15/12.5	–0.60 /12.5	–0.95/12.5
2163	–0.63/50	2.10/50	–0.26/50	**5.19/50**
2164	**6.69/25**	1.50/50	**10.91/50**	**21.14/50**
2165	–1.47/0.8	1.13/12.5	**7.21/6.25**	2.33/0.8
2166	**11.14/50**	1.30/50	4.60/50	**40.33/50**
2167	**27.23/25**	0.75/50	**13.11/50**	**33.44/50**
2168	–1.25/50	–0.44/50	0.22/50	**9.10/50**
2169	4.17/50	–0.82/25	–1.61/50	**5.98/50**
2173	0.71/0.8	1.80/50	**21.12/25**	**68.33/12.5**
2174	2.42/12.5	0.57/12.5	0.72/12.5	**11.31/12.5**
2175	–2.28/2.5	–0.09/2.5	0.90/2.5	–1.18/2.5
2176	–1.91/50	–3.85/50	–0.54/50	–2.77/50
2177	–1.04/5	–0.20/5	–0.23/5	–1.35/5
2178	0.81/1.6	0.36/12.5	1.01/12.5	**10.38/0.8**
2182	–1.24/2.5	0.27/2.5	–0.29/2.5	1.79/2.5
2185	3.97/12.5	0.14/12.5	0.35/12.5	**8.76/12.5**
2186	0.22/2.5	0.09/2.5	–0.59/2.5	–2.01/2.5

Difference (Δ) in fluorescence compound -Control (no compound)/ concentration at ½ MIC (mg/L). Bacteria were incubated at 37˚C in PBS (pH 7.4) containing 1 mg/L EB and with and without the phenothiazine derivative at ½ of its MIC. The difference in the amount of fluorescence between the compound-containing culture and its control (no compound) is presented. The variation of amount of fluorescence of controls from experiment to experiment yielded a standard deviation of ±2.5 relative fluorescence units. A positive difference of 5 or more fluorescence units is deemed significant and demonstrates an increase of retained EB due to an effect on the efflux pump system of the bacterium by the compound. Compounds deemed effective inhibitors and the resulting amount of increased relative fluorescence are highlighted in bold.

tetracycline (3), *Staphylococcus aureus* COL_{OXA} that over-expresses the NorA efflux pump and is extremely resistant to oxacillin (6, 18) and two strains of *Salmonella* Enteritidis that overexpress the AcrAB efflux pump by 6 times that of their parental stains and have been induced to high level resistance to ciprofloxacin (19). Because these efflux pump-mediated MDR bacteria can be made more sensitive to antibiotics to which they were resistant by an EPI, such as TZ, that inhibits their efflux pumps, this phenothiazine was employed as the positive control. Briefly, none of the compounds at their highest concentration reduced the MIC of antibiotics for the Gram-negative bacteria; only compound 2173 reduced the resistance of the Gram-positive bacteria to oxacillin.

Discussion

The results of this study show that whereas many of the compounds have moderate to very potent EPI activity against the efflux pumps of Gram-positive pathogenic bacteria, fewer compounds have EPI-like activity against *Escherichia coli* and only one, compound 2158, has potent activity against *Salmonella* Enteritidis. These results are consistent with the general response of efflux pumps to EPIs in that the efflux pumps of Gram-positive bacteria are significantly more affected by EPI like agents (26). With the exception of *Salmonella* Enteritidis, compound 2173 had activity against the bacteria employed in this study. However, its ability to significantly reduce efflux-mediated resistance was restricted to *Staphylococcus aureus* strains that over-expressed the efflux pump NorA (COL_{OXA} and HPV 107 strains). Consequently, if 2173 is devoid of any toxicity, it may have some potential for the therapy of efflux-mediated MDR Staphylococcus strains. Moreover, because the vast majority of MRSA strains have an MDR phenotype (27-29), compound 2173 has importance. It must be mentioned that although many compounds had activity against the efflux pump of wild type *Enterococcus faecalis*, none reduced its resistance to the antibiotic (data not shown); the inability to reduce resistance lies in the intrinsic basal resistance of this species to the antibiotic.

Salmonella sp. has many responses to noxious agents that are invoked subsequent to exposure. Among these is the activation of genes that regulate and code for the main efflux pump of the organism, the AcrAB pump (14, 19, 30). As an example, exposure of Salmonella sp. to concentrations of chlorpromazine at the MIC or multiples of the MIC, results in the over-expression of the AcrAB pump (30). However, when exposed to sub-inhibitory concentrations of chlorpromazine, the organism at first is susceptible to the agent for the first four to six hours of exposure, and then gradually becomes resistant (31). This response to chlorpromazine has also been observed with exposure of Salmonella sp. to sub-inhibitory concentrations of TZ (14). In addition, during the first four to six hours of exposure, there is a sequential activation of the stress gene *soxS*, followed by the global regulator *ramA*, then by the local regulator *marA*, and lastly, *acrB* (14). These results suggest that the EPI-like activity of compound 2158 in the EB efflux assay, an assay that does not support growth since ions required for growth (K^+, Ca^{2+}, *etc*.) are absent, would soon be followed in complete medium by the genetic events associated with progression of Salmonella sp. from a status of susceptibility to one of extreme resistance (MIC >200 mg/L) to the phenothiazine. Such studies are now on-going.

In conclusion, the evaluation of forty phenothiazines derived from a single phenothiazine parent for EPI activity against pathogenic bacteria whose resistance to given antibiotics is mediated by over-expression of efflux pumps suggests that compound 2173, due to its ability to reduce resistance of MRSA strains to oxacillin, has potential as an adjunct agent to oxacillin for therapy of MDR infections, provided that it is relatively toxic-free at concentrations that are effective. Compound 2158 deserves further study inasmuch as the compound has significant EPI-like activity against the efflux pump of *Salmonella* Enteritidis.

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